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Microbiome Diversity in the Bronchial Tracts of Patients with Chronic Obstructive Pulmonary Disease

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Culture of bacteria from bronchial secretions in respiratory patients has low sensitivity and does not allow for complete assessment of microbial diversity across different bronchial compartments. In addition, a significant number of clinical studies are based on sputum samples, and it is not known to what extent they describe the real diversity of the mucosa. In order to identify previously unrecognized lower airway bacteria and to investigate the complexity and distribution of microbiota in patients with chronic obstructive pulmonary disease (COPD), we performed PCR amplification and pyrosequencing of the 16S rRNA gene in patients not showing signs or symptoms of infection. Four types of respiratory samples (sputum, bronchial aspirate, bronchoalveolar lavage, and bronchial mucosa) were taken from each individual, obtaining on average >1,000 16S rRNA sequences per sample. The total number of genera per patient was >100, showing a high diversity, with *Streptococcus*, *Prevotella*, *Moraxella*, *Haemophilus*, *Acinetobacter*, *Fusobacterium*, and *Neisseria* being the most commonly identified. Sputum samples showed significantly lower diversity than the other three sample types. Lower-bronchial-tree samples, i.e., bronchoalveolar lavage and bronchial mucosa, showed a very similar bacterial compositions in contrast to sputum and bronchial aspirate samples. Thus, sputum and bronchial aspirate samples are upper bronchial tree samples that are not representative of the lower bronchial mucosa flora, and bronchoalveolar lavage samples showed the results closest to those for the bronchial mucosa. Our data confirm that the bronchial tree is not sterile in COPD patients and support the existence a different microbiota in the upper and lower compartments.

The bronchial tree and the pulmonary parenchyma are considered sterile in healthy subjects, but potentially pathogenic microorganisms (PPM) are often recovered from bronchial secretions in patients with chronic obstructive pulmonary disease (COPD) during periods of clinical stability and, particularly, during exacerbations, when bacterial loads increase significantly (37, 46). When sputum has been used for the identification of bronchial colonization by PPM in stable COPD, positive cultures have been found in one-fifth to three quarters of the patients, in most cases to a single microorganism (16, 38). Cultures positive for PPM have been found in one-third of the patients with COPD in the absence of symptoms of bronchial infection when lower bronchial secretions have been sampled, avoiding the oropharynx through the use of a protected specimen brush for the collection of the specimens under sterile conditions, with *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Mycobacterium catarrhalis* being the bacteria most often recovered (37), a finding that confirms the presence of these microorganisms in the lower bronchial tree of COPD patients. Bronchial colonization has been also demonstrated in bronchoalveolar lavage samples obtained from COPD patients, a sample that recovered peripheral secretions from the bronchial tree (8, 39, 42). Most PPM cultured from stable COPD patients show low microbial loads (<100,000 CFU/ml), however, and are not associated with a neutrophilic inflammatory response (31, 45), with high loads being found almost exclusively when *H. influenzae* is the colonizing bacteria (25, 26). These findings confirm that colonizing PPM may be found in the bronchial trees of some patients with COPD, in most cases as a single culture. The mechanisms behind the recovery of low-load PPM from bronchial secretions in patients with COPD in the absence of signs and symptoms of infection is open to debate, since it may be related to oropharyngeal bacteria that migrated to the bronchial tree or to flora colonizing specifically the lower bronchial tree (30, 44).

Culture-based techniques underdiagnose bronchial colonization at loads below the detection limit of the sputum culture, and PPM have been identified in one-tenth of culture-negative sputum samples (28). The use of molecular methods such as PCR amplification of the 16S rRNA gene, followed by cloning and traditional Sanger sequencing in bronchial secretion samples, has allowed the identification of bacterial species previously undetected by the selective cultures used for the identification of PPM and, more recently, the application of pyrosequencing to PCR-amplified products from human samples has taken the study of microbial diversity to an unprecedented level of detail (7, 14, 17). These approaches have shown that there is a wide diversity of microorganisms in respiratory secretions that remain undetected in culture (15) and have suggested that the bronchial microbiome may be heterogeneous in COPD, with significant differences between bronchial sections (14), a finding previously reported in

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protected specimen brush samples from patients with respiratory disease (20) and not observed in healthy subjects (7). Accordingly, before the initiation of large studies focusing on the microbiome of the bronchial tree in well-characterized COPD patients, it is necessary to examine the microbial diversity across its different sections, so that the interpretation of the results obtained through sampling of the different bronchial tree compartments is accurate.

The aim of the present study was to identify lower-airway bacteria unrecognized through culture in patients with COPD, in the absence of signs and symptoms of bronchial infection, as well as to examine the complexity of microbial flora in these patients by the use of amplification and pyrosequencing of the 16S rRNA gene. In addition, we sought to compare microbial diversity recovered from upper (sputum and bronchial aspirate) and lower (bronchoalveolar lavage and bronchial mucosa) bronchial tree samples simultaneously obtained from the same individuals, under the assumption that these samples represent different bronchial tree compartments.

MATERIALS AND METHODS

Design and participants. A study focused on the microbiome of the bronchial tree in COPD was performed in stable patients with moderate disease who had not suffered from exacerbations during the previous year and had not been treated with antibiotics during this period. Patients with sputum cultures available and negative for PPM at enrollment and at least two times in the previous year were recruited between January and June 2010. Gram-negative and Gram-positive bacteria recognized as agents causing respiratory infections, such as *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterobacteriaceae* were considered as PPM (6, 29, 40). Patients with severe lung function impairment (postbronchodilator forced expiratory volume in 1 s [FEV_1] at 50% of the reference) or requiring regular treatment for chronic respiratory disease were excluded from the study, and additional exclusion criteria included hospitalization within the previous year and any severe disease needing regular therapy. Patients surgically treated for cancer and free of recurrence for a minimum of 5 years were accepted for inclusion. All patients gave written informed consent, and the study protocol was approved by the Regional Ethics Committee. Four types of respiratory samples (sputum, bronchial aspirate, bronchoalveolar lavage, and bronchial mucosa) were taken from each individual.

Sociodemographic and clinical measurements. Sociodemographic and clinical data were recorded at enrollment, including smoking habits, medical antecedents, respiratory symptoms, and treatments. All patients performed forced spirometry and reversibility tests in the morning with a dry rolling-seal spirometer (Spirometrics, Gray, ME) according to standard techniques (2). Postbronchodilator forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1) were measured and compared to age and height-adjusted reference values obtained from selected volunteers from the Barcelona province (36). The data were analyzed using the SPSS statistical software package, version 18 (SPSS, Inc., Chicago, IL). The results of descriptive statistics for categorical variables are expressed as absolute and relative frequencies. The results for continuous variables are expressed as medians (i.e., interquartile range).

Sampling procedure. Induced sputum samples were obtained and processed within 60 min at the enrollment visit according to standard methods (32, 33). Briefly, the patient was pretreated with an inhaled b2-agonist 10 min before the nebulization of isotonic saline (0.9%), followed by increasing concentrations of hypertonic saline (3, 4, and 5%) for 7 min with each concentration. After every induction, the patient attempted to obtain a sputum sample by coughing, and the nebulization procedure was discontinued when the sputum volume collected was $\geq$1 ml (1).

The sputum sample was cultured and the determination of microbial typology was carried out by means of culture in selective media according to standard methods (4), and cultures were considered negative when not growing PPM. Bronchoscopy was performed under local anesthesia and sedation, using a flexible videobronchoscope (BF180; Olympus Optical Co., Tokyo, Japan). Local anesthesia and sedation were achieved using topical lidocaine spray and intravenous midazolam, respectively, in accordance with standard recommendations (5, 35). A bronchoalveolar lavage, a bronchial mucosa biopsy specimen, and a bronchial aspirate were collected through the working channel of the bronchoscope during the procedure. The bronchoscope, after its usual disinfection procedure (5), was introduced transnasally, passed through the vocal cords without aspiration and wedged in a right middle lobe bronchus for bronchoalveolar lavage, to avoid contamination of the collected sample by oropharyngeal flora (7). Saline (50 ml) was instilled, aspirated, and discarded, after which 100 ml was lavaged in the same location and collected, with recovery of a volume >30% of the instilled sample. Subsequently, a bronchial biopsy was performed in a subsegmentary bronchus macroscopically normal at white light examination, and bronchial aspirates with the tip of the bronchoscope located in the right and left main bronchi were obtained.

Sample processing and DNA extraction. Samples were collected between April and August 2009 and kept at −80°C until DNA extractions were performed in September 2009. Sputum, bronchial aspirate, and bronchoalveolar lavage samples were treated for 15 min with an equal volume of Sputasol (Oxoid, Hampshire, United Kingdom), followed by centrifugation for 15 min at 13,000 × *g*. Genomic DNA extraction from sputum, bronchial aspirate, and bronchoalveolar lavage pellets, and bronchial mucosa samples was performed using a Qiagen DNA blood kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's instructions, with some modifications. Briefly, samples were treated with 10 μl of an in-house lysis solution as previously described (11). This stock solution consisted of 10 ml of filter-sterilized buffer 1 (20 mM Tris [pH 8], 2 mM EDTA [Sigma] 1.2% Triton X-100), 500 mg of lysozyme (Sigma, Poole, United Kingdom), 50,000 U of mutanolysin (Sigma), and 1,000 U of lysostaphin (Sigma). The amounts of DNA obtained ranged between 80 and 950 ng for sputum, 70 and 1,105 ng for bronchial aspirates, 164 and 1,060 ng for bronchoalveolar lavages, and 70 and 520 ng for bronchial mucosa samples.

PCR amplification and pyrosequencing. The first 500 bp of the 16S rRNA genes were amplified with the universal eubacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 338R (5′-GCCTTGCCAG CCCGCTCAGGC-3′) using the high-fidelity AB-gene DNA polymerase (Thermo Scientific) with an annealing temperature of 52°C and 20 cycles to minimize PCR biases (41). A secondary amplification was performed using the purified PCR product as a template, in which the universal primers were modified to contain the pyrosequencing adaptors A and B, and an 8-bp "barcode" specific to each sample, following McKenna and cols (27). Barcodes were different in at least three nucleotides from each other to avoid misclassification in sample assignments. Five secondary PCRs were performed per sample, pooling their PCR products before purification, which was done using an Ultrapure PCR purification kit (Roche). The final DNA per sample was measured by PicoGreen (Invitrogen) fluorescence in a Modulus 9200 fluorimeter from Turner Biosystems, and 12 samples were mixed in equimolar amounts. Each pool of 12 samples was further purified and concentrated by the use of Microcon filters (Millipore) into a final volume of 20 μl and a concentration higher than 100 μg/μl. PCR products were pyrosequenced from the forward primer end only using a GS-FLX sequencer with titanium chemistry (Roche) at the Center for Genomic Regulation in Barcelona, Spain. One-sixteenth of a plate was used for each pool of 12 samples.

Sequence analysis. Reads with an average quality value lower than 20 and/or with more than four ambiguities in homopolymeric regions in the first 360 flows were excluded from the analysis. Only reads longer than 200 bp were considered, since it has been shown that taxonomic assignment accuracy decreases dramatically in reads shorter than 200 bp and that the use of short reads inflate rarefaction curves (9). Chimeric sequences were filtered out using the software Bellerophon (19). Sequences with differ-

TABLE 1 Characteristics of the studied population

Patient	Gender	Smoking	Cancer history	Cancer surgery	COPD severity[a]	FEV_1[b] (%)	No. of sequences[c]
1	Male	Former	Lung	Lobectomy	Moderate	72	4,362
2	Female	Former	Breast	Mastectomy	Moderate	76	2,477
4	Male	Former	Lung	Lobectomy	Moderate	41	2,980
5	Male	Former	None		Moderate	60	6,858
7	Male	Former	None		Moderate	72	5,497
8	Male	Former	Lung	Lobectomy	Moderate	42	2,619

[a] According to GOLD criteria (www.goldcopd.org).
[b] FEV_1, forced expiratory volume in 1 s.
[c] Sequences of the 16S rRNA gene obtained after length and quality filtering.

ences in the primer region were excluded from the analysis, as well as sequences with more than four ambiguities in homopolymeric regions. Sequences were assigned to each sample by the 8-bp barcode and passed through the Ribosomal Database Project classifier (10), where each read was assigned a phylum, class, family, and genus, as long as the taxonomic assignment was unambiguous within an 80% confidence threshold, which has been estimated to assign reads with >95% accuracy at those taxonomic levels. Sequences were deposited in the MG-RAST server under accession numbers 4481640.3 to 4481663.3. To estimate the total diversity, sequences were clustered at 97% nucleotide identity over 90% sequence alignment length using the software CD-HIT (22), and rarefaction curves were obtained with the program Analytic Rarefaction 1.3 (18). For this analysis, sequences >97% identical were considered to correspond to the same operational taxonomic units, representing a group of reads that likely belong to the same species (47). Rarefaction curves were also obtained using only those sequences assigned to a genus by the RDP classifier, as a conservative estimate of diversity at that taxonomic level.

Statistical analysis. Principal component analyses (PCA) were performed with UNIFRAC (24) using clustering at 97% sequence identity with the weighted analysis option, which compares the 16S-estimated diversity by a phylogenetic approach that takes into account both taxonomically assigned and unassigned reads. For comparison to respiratory tract samples, PCR-amplified sequences from the same region of the 16S rRNA gene in oral samples from supragingival dental plaque were taken (MG-Rast accession numbers 4481871.3 to 4481856.3) and included in the PCA. The matrix for performing the PCA is based on the distance between phylogenetic trees corresponding to each sample. This distance is measured in terms of the branch lengths in the trees that are unique to one sample or the other (24).

Two-way comparisons in bacterial composition were performed using the UniFrac metric (24) in order to measure whether the microbial communities in different sample types were significantly different. A tree including all sequences from each sample type was obtained, and samples are considered significantly different if the UniFrac value for the real tree is greater than would be expected if the sequences were randomly distributed between the samples. The sample identifications are randomly permuted 1,000 times to obtain a *P* value representing the fraction of permuted trees that have UniFrac values greater than or equal to that of the real tree, using Bonferroni corrections for multiple comparisons (24).

RESULTS

Participants. Five men and one woman who were former smokers with moderate COPD were included in the study (median age, 71 years). Four patients reported chronic bronchitis and all patients showed a moderate impairment in their lung function (postbronchodilator FEV_1 median, 66% of the reference) that did not require regular treatment. Four patients had been surgically treated for cancer (three lung cancer patients and one breast cancer patient), all of them free of recurrence of this disease after surgery for a minimum of 5 years. Sputum cultures obtained at recruitment and on two occasions during the previous year were negative for PPM. Clinical data for the study group are shown in Table 1.

Microbial diversity in the bronchial tree. An average of 1,033 sequences of the 16S rRNA gene were obtained in the 24 respiratory samples, which correspond to sputum, bronchial aspirate, bronchoalveolar lavage, and bronchial mucosa from the six participating COPD patients. When sequences were clustered at 97% sequence identity, a consensus threshold for reads belonging to the same species, rarefaction curves, and Chao1 indices suggested a total diversity of more than 500 species per sample.

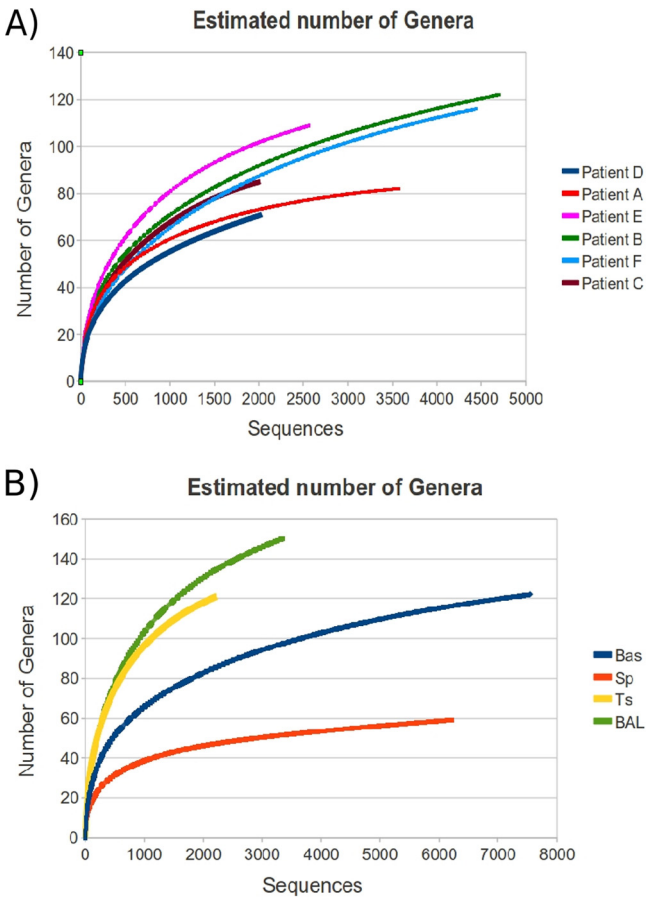

FIG 1 Bacterial diversity in respiratory tract samples. The graph shows rarefaction curves indicating the number of assigned bacterial genera in relation to the number of 16S rRNA sequences, grouped by individual (A) and sampling method (B). Abbreviations: Sp, sputum; Ts, tissue; BAL, bronchoalveolar lavage; Bas, bronchial aspirate.

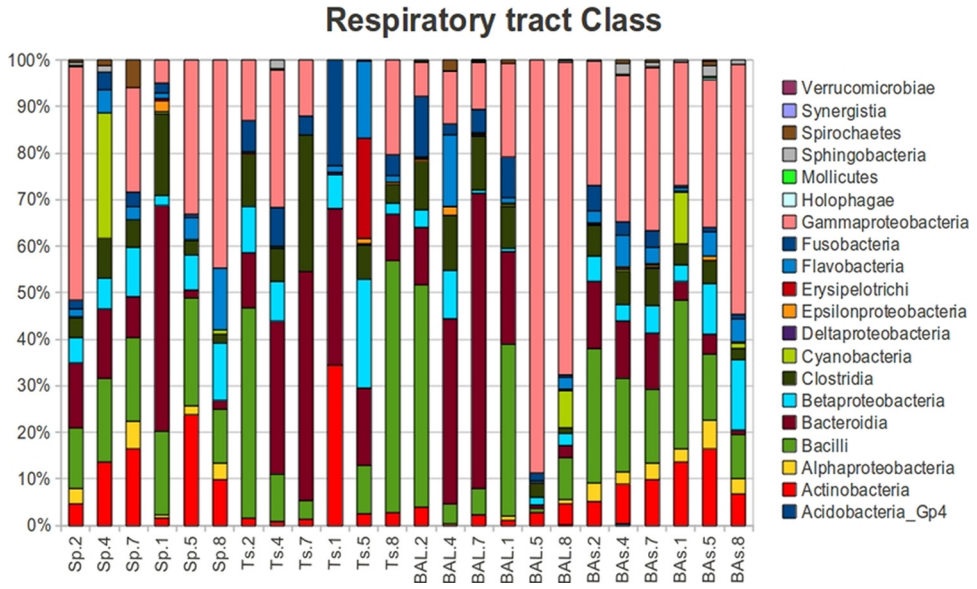

FIG 2 Taxonomic assignment of the 24 samples at the level of bacterial classes. The bacterial composition in sputum samples appears to be particularly different from other sample types from the same individual. Abbreviations: Sp, sputum; Ts, tissue; BAL, bronchoalveolar lavage; BAs, bronchial aspirate.

Rarefaction curves at the genus level reached saturation for the subjects with lower microbial diversity when the sequences of the four sample types from each patient were pooled, and the total number of bacterial genera was found to be between 80 and 140 per patient, with differences in the bacterial composition among them (Fig. 1A). Commonly amplified bacterial phyla were *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*. *Streptococcus*, *Prevotella*, *Moraxella*, *Haemophilus*, *Acinetobacter*, *Fusobacterium*, and *Neisseria* were the most common bacterial genera amplified, which together account for 60% of the total number of sequences. A complete list of bacterial genera per sample is included in Data Set S1 in the supplemental material. Sequences with maximum identity to *Legionella* and *Mycoplasma* were also identified in the samples. Although the short sequence lengths of the reads makes assigning species unreliable, further analysis of these sequences against the RDP database suggests that they probably correspond to *Legionella dresdenensis*, a species not associated with clinical cases isolated from river water, and the nonpathogenic species *Mycoplasma orale* and *M. salivarium*.

Microbiome differences in bronchial tree compartments. The bacterial composition was similar for the same sample type (Fig. 2), whereas important differences in diversity were observed between upper and lower bronchial samples. The bacterial diversity was lower in sputum, which reached saturation at about 60 bacterial genera, and much higher in bronchoalveolar lavage and biopsy specimens (Fig. 1B and 2). Some of the most frequent genera in sputum and in bronchial aspirate correspond to common dwellers of the oral cavity, including *Veillonella*, *Fusobacterium*, or *Prevotella* spp.

In order to test differences in bacterial composition among the four sample types, a PCA was performed for all 16S rRNA reads clustered at 97% similarity, giving a higher resolution than genera assignment and taking into account all species-level phylotypes. The two main components accounted for over 83% of data variation, and the graph shows that bronchial mucosa and bronchoalveolar lavage samples cluster together, whereas sputum and bronchial aspirate samples were distinct from the lower bronchial samples and were different between them (Fig. 3). This was confirmed by two-way comparisons in microbial composition, which showed statistically significant differences between sputum and the other sample types (UniFrac distance, $P < 0.002$ in all cases),

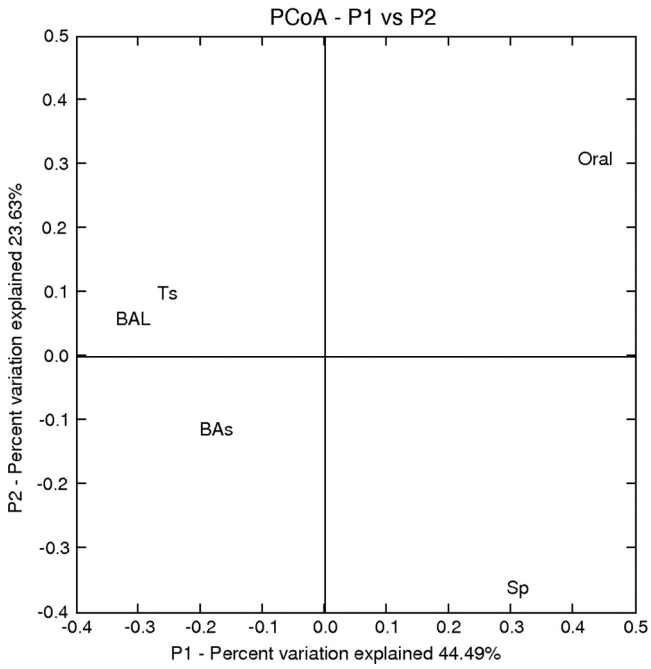

FIG 3 Principal component analysis of the four respiratory tract sample types ($n = 6$ for each sample type) and samples from the oral cavity of healthy individuals ($n = 16$) according to the microbial composition, as inferred by pyrosequencing of the 16S rRNA gene. Similar results were obtained using the third component (data not shown). Abbreviations: Sp, sputum; Ts, tissue; BAL, bronchoalveolar lavage; BAs, bronchial aspirate; Oral, supragingival dental plaques.

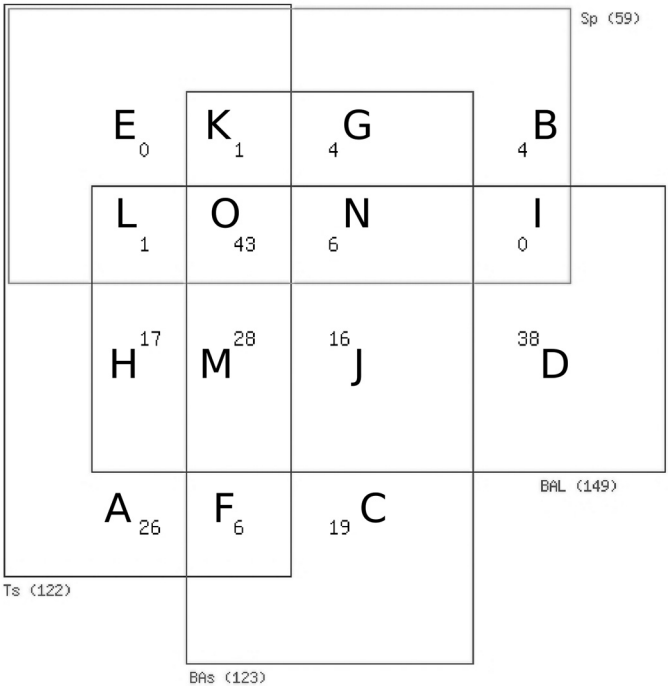

FIG 4 Venn diagram showing the bacterial genera unique and/or shared between sample types. A complete list of genera for each location in the diagram is included in Table S1 in the supplemental material. Abbreviations: Sp, sputum; Ts, tissue; BAL, bronchoalveolar lavage; BAs, bronchial aspirate.

whereas bronchoalveolar lavage and biopsy specimens were not statistically different (UniFrac distance, $P = 0.6$). The similarity between the microbiomes of bronchoalveolar lavage and bronchial mucosa suggest that both samples represent the same bronchial compartment. Bronchial aspirates and sputum are less diverse and contain genera absent from the lower bronchial tree but which are found in the oral cavity of healthy individuals. Figure 4 shows the genera shared between, and unique to each bronchial tree sample, indicating that 43 genera are shared among all sample types (see Table S1 in the supplemental material for a list of genera unique to and shared between samples). Although a large number of genera appear to be unique to each sample type, most of them are detected as single reads in the sequences. When these singletons are excluded (see Data Set S1 in the supplemental material), the genera exclusive of each sample type are restricted to *Limnobacter* in sputum, to *Arcobacter*, *Blautia*, *Emticicia*, and *Runella* in bronchial aspirate, and to *Azonexus*, *Herbaspirillum*, *Peredibacter*, *Simplicispira*, *Sporolactobacillus*, and *Methylobacillus* in bronchoalveolar lavage.

DISCUSSION

Our study has analyzed the bronchial tree microbiome in stable COPD patients with a moderate impairment in their lung function, through amplification and pyrosequencing of the 16S rRNA gene. We have characterized the microbiology of upper and lower bronchial tree compartments, examining different respiratory samples that included sputum, bronchial aspirate, bronchoalveolar lavage, and bronchial mucosa. Rarefaction curves and Chao1 indices demonstrated a number of 80 to 140 bacterial genera per patient, with a total estimated diversity of over 500 species in the examined samples. The number of potential species in the respiratory samples could be inflated due to sequencing errors (34) and short sequence length attributable to the coverage of the hypervariable regions 1 and 2 of the 16S rRNA gene by the read. Taxonomic assignment at higher levels such as genus or family, however, is highly reliable at this read length (23) and confirms the presence of a complex bacterial community. Thus, bacterial diversity in the lower airway, at least in COPD patients without signs of infection, is much higher than previously anticipated. Frequently amplified phylum in these patients were *Proteobacteria*, *Bacteroidia*, *Actinobacteria*, and *Firmicutes*, with *Streptococcus*, *Prevotella*, *Moraxella*, *Haemophilus*, *Acinetobacter*, *Fusobacterium*, and *Neisseria* being the most common bacterial genera identified, which together account for 60% of the total number of sequences. The bacterial diversity was lower in sputum, a sample type that reached saturation at about 60 bacterial genera, and much higher in bronchoalveolar lavage and bronchial mucosa specimens. The microbiomes of these lower bronchial tree samples showed close similarity, whereas sputum and bronchial aspirate were distinct between them and from the lower samples.

Traditional culture-based studies have described the bronchial tree as sterile in healthy subjects (21, 37, 43), recovering bacteria from bronchial secretions only when the patient suffers from a chronic respiratory disease. In the absence of signs or symptoms of respiratory infection, low-load colonizing PPM are often found in the bronchial tree of COPD patients (25, 26, 37), being the mechanisms behind the recovery of these microorganisms in COPD open to debate. Culture-independent microbiological techniques have demonstrated that the lungs are not sterile during health and have documented changes in the lung microbiome in several chronic lung diseases (3). Charlson et al. have studied the oropharyngeal and bronchial secretions from healthy subjects, finding close similarities in the microbiologic pattern found in the oropharynx and the bronchial tree, with a lower biomass in bronchial secretions, and concluded that a specific bronchial microbiome in healthy subjects does not exist (7). A different pattern was reported by Hilty et al. in patients with COPD (17). These authors compared lower bronchial secretions recovered by bronchial brushing from healthy subjects and patients with COPD and asthma and demonstrated a frequent recovery of members of the phylum *Proteobacteria*, that contained important PPM-like *Haemophilus* and *Moraxella* in these patients. Erb-Downward et al. (14) used a similar approach in smokers without functional abnormalities and with COPD using bronchoalveolar lavage to sample the lower bronchial tree and found a close similarity in the microbiome of smokers with or without disease, who showed members of the phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Fusobacteria* in over a half of the patients and a similar diversity, with *Streptococcus*, *Prevotella*, *Fusobacterium*, *Pseudomonas*, *Haemophilus*, *Veillonella*, and *Porphyromonas* being the most commonly identified genera. These authors also found a different bacterial profile, with an overrepresentation of the genus *Pseudomonas*, in severe COPD patients, when explants of the bronchial mucosa obtained from surgical samples were examined (14). An important limitation of these studies performed in COPD patients is that the microbial diversity in the different bronchial compartments has not been assessed, making the interpretation of the results difficult.

Our study focused on moderate COPD patients and confirmed that in the absence of signs and symptoms of bronchial infection

there is a rich microbiome in the bronchial tree, the most common amplified bacterial phylum being *Proteobacteria*, *Bacteroidia*, *Actinobacteria*, and *Firmicutes*, which have been reported in healthy subjects too (7). In the studied samples, common bacterial genera amplified have been *Streptococcus*, *Prevotella Fusobacterium*, and *Neisseria*, also described in the normal population (7), and *Moraxella*, *Haemophilus*, and *Acinetobacter*, bacteria that have appeared as overrepresented in patients with COPD (14, 17). Accordingly, our data support the hypothesis that in patients with COPD the bronchial microbiome includes genera present in the healthy subjects, with an additional increased presence of various genera of *Proteobacteria* that are unusual in the normal population and included well-known PPM such as *Haemophilus* and *Moraxella*.

Clear-cut differences in the microbiome of the upper and lower bronchial tree of moderate COPD patients emerged from our study. The upper respiratory samples, sputum and bronchial aspirate, showed low diversity and the frequent recovery of phyla that are part of the oropharyngeal flora of the healthy subject, such as *Firmicutes* and *Bacteroidetes* (7). Lower-bronchial-tree samples (bronchoalveolar lavage and bronchial biopsy specimens) showed a more diverse microbiome with a close community profile in both samples, a minor representation of oropharyngeal flora, and the recovery of genera that included PPM. These results confirm that the bronchial tree has different compartments with specific characteristics in COPD. The upper bronchial tree has low diversity and an overrepresentation of oropharyngeal flora, and lower bronchi show a higher diversity that included genera that are unusual in sputum and aspirates and only show low prevalence of *Firmicutes* and *Bacteroidetes*, which are common in the mouth and pharynx.

The lower level of bacterial diversity found in sputum compared to other sample types suggests that sputum samples contain a limited fraction of the total bacterial community inhabiting the respiratory tract. In addition, PCA indicates that sputum samples, which are commonly used for bacterial identification in respiratory tract infections, are not representative of the composition and proportion of bacterial taxa in bronchial mucosa. Bronchoalveolar lavage, on the other hand, showed a similar but slightly higher diversity than mucosa, probably attributable to the wider bronchial surface sampled by lavage, and may be considered a representative substitute of bronchial mucosa samples (Fig. 1B). The representativeness of sampling methods is a common problem in human microbiome research. Most studies of microbial diversity in the gastrointestinal tract, for instance, have been performed on stool samples, but gut mucosal biopsy specimens have, in fact, been found to harbor a very different microbial composition compared to fecal samples of the same individuals (13), questioning the validity of many metagenomic studies. Similarly, the use of inappropriate samples to study the lung's bacterial diversity can also have important clinical implications. Given that sputum samples are probably the most common lung clinical samples taken because they are readily obtained with noninvasive techniques, it must be born in mind that the microbiota of sputum is not representative of the microbiology of the lower airway. Thus, for example, we did not detect *Legionella* in sputum, but it did appear in four of six bronchoalveolar lavage samples. The role of atypical bacteria such as *Legionella* in COPD is unclear and should be further studied, since other authors have detected *Legionella* in COPD patients by PCR methods (12).

Finally, we want to emphasize that although we cannot discard that the sharing of some bacterial genera between upper bronchial tree samples and oral cavity can be partly attributed to some bacterial biomass contamination at the time of sampling (7), the observed resemblance may also have a biological meaning. The larynx has classically been considered a barrier between the oropharynx and the trachea, which would keep the latter sterile. However, the data presented here suggest that bacteria from the oral cavity and the pharynx are also found in the bronchial tree, and such mechanical barrier does not avoid a regular appearance of oropharyngeal flora in the upper bronchi, which decreases when going further down in the airway, at the level where the bronchoalveolar lavage is performed. We hope these results stimulate further characterization of the respiratory tract microbiota in healthy controls and in individuals with different respiratory diseases.

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